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(54) Title: ANTISENSE MODULATION OF APOLIPOPROTEIN B EXPRESSION

(57) Abstract: Antisense compounds, compositions and methods are provided for modulating the expression of apolipoprotein B. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding apolipoprotein B. Methods of using these compounds for modulation of apolipoprotein B expression and for treatment of diseases associated with expression of apolipoprotein B are provided.



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(54) Title: ANTISENSE MODULATION OF APOLIPOPROTEIN B EXPRESSION

(57) Abstract: Antisense compounds, compositions and methods are provided for modulating the expression of apolipoprotein B. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding apolipoprotein B. Methods of using these compounds for modulation of apolipoprotein B expression and for treatment of diseases associated with expression of apolipoprotein B are provided.



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**ANTISENSE MODULATION OF APOLIPOPROTEIN B EXPRESSION**

This application claims priority of U.S. provisional Application Serial No: 60/426,234, filed November 13, 2002, and is a continuation-in-part of U.S. Application Serial No. 10/147,196 filed May 15, 2002 (Attorney Docket No. ISPH-0664) which is a continuation-in-part of U.S. Application Serial No. 10/135,985 filed April 30, 2002 (Attorney Docket No. ISPH-0663) which is a continuation-in-part of U.S. Application Serial No. 09/920,033 filed August 1, 2001 (Attorney Docket No ISPH-0592).

**FIELD OF THE INVENTION**

The present invention provides compositions and methods for modulating the expression of apolipoprotein B. In particular, this invention relates to compounds, particularly oligonucleotides, specifically hybridizable with nucleic acids encoding apolipoprotein B. Such compounds have been shown to modulate the expression of apolipoprotein B.

**BACKGROUND OF THE INVENTION**

Lipoproteins are globular, micelle-like particles that consist of a non-polar core of acylglycerols and cholesteryl esters surrounded by an amphiphilic coating of protein, phospholipid and cholesterol. Lipoproteins have been classified into five broad categories on the basis of their functional and physical properties: chylomicrons, which transport dietary lipids from intestine to tissues; very low density lipoproteins (VLDL); intermediate density lipoproteins (IDL); low density lipoproteins (LDL); all of which transport triacylglycerols and cholesterol from the

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liver to tissues; and high density lipoproteins (HDL), which transport endogenous cholesterol from tissues to the liver.

Lipoprotein particles undergo continuous metabolic processing and have variable properties and compositions. Lipoprotein densities increase without decreasing particle diameter because the density of their outer coatings is less than that of the inner core. The protein components of lipoproteins are known as apolipoproteins. At least nine apolipoproteins are distributed in significant amounts among the various human lipoproteins.

Apolipoprotein B (also known as ApoB, apolipoprotein B-100; ApoB-100, apolipoprotein B-48; ApoB-48 and Ag(x) antigen), is a large glycoprotein that serves an indispensable role in the assembly and secretion of lipids and in the transport and receptor-mediated uptake and delivery of distinct classes of lipoproteins. The importance of apolipoprotein B spans a variety of functions, from the absorption and processing of dietary lipids to the regulation of circulating lipoprotein levels (Davidson and Shelness, *Annu. Rev. Nutr.*, 2000, 20, 169-193). This latter property underlies its relevance in terms of atherosclerosis susceptibility, which is highly correlated with the ambient concentration of apolipoprotein B-containing lipoproteins (Davidson and Shelness, *Annu. Rev. Nutr.*, 2000, 20, 169-193).

Two forms of apolipoprotein B exist in mammals. ApoB-100 represents the full-length protein containing 4536 amino acid residues synthesized exclusively in the human liver (Davidson and Shelness, *Annu. Rev. Nutr.*, 2000, 20, 169-193). A truncated form known as ApoB-48 is colinear with the amino terminal 2152 residues and is synthesized in



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the small intestine of all mammals (Davidson and Shelness, *Annu. Rev. Nutr.*, 2000, 20, 169-193).

ApoB-100 is the major protein component of LDL and contains the domain required for interaction of this lipoprotein species with the LDL receptor. In addition, ApoB-100 contains an unpaired cysteine residue which mediates an interaction with apolipoprotein(a) and generates another distinct atherogenic lipoprotein called Lp(a) (Davidson and Shelness, *Annu. Rev. Nutr.*, 2000, 20, 169-193).

In humans, ApoB-48 circulates in association with chylomicrons and chylomicron remnants and these particles are cleared by a distinct receptor known as the LDL-receptor-related protein (Davidson and Shelness, *Annu. Rev. Nutr.*, 2000, 20, 169-193). ApoB-48 can be viewed as a crucial adaptation by which dietary lipid is delivered from the small intestine to the liver, while ApoB-100 participates in the transport and delivery of endogenous plasma cholesterol (Davidson and Shelness, *Annu. Rev. Nutr.*, 2000, 20, 169-193).

The basis by which the common structural gene for apolipoprotein B produces two distinct protein isoforms is a process known as RNA editing. A site specific cytosine-to-uracil editing reaction produces a UAA stop codon and translational termination of apolipoprotein B to produce ApoB-48 (Davidson and Shelness, *Annu. Rev. Nutr.*, 2000, 20, 169-193).

Apolipoprotein B was cloned in 1985 (Law et al., *Proc. Natl. Acad. Sci. U. S. A.*, 1985, 82, 8340-8344) and mapped to chromosome 2p23-2p24 in 1986 (Deeb et al., *Proc. Natl. Acad. Sci. U. S. A.*, 1986, 83, 419-422).

Disclosed and claimed in US patent 5,786,206 are methods and compositions for determining the level of low density lipoproteins (LDL) in plasma which include isolated DNA sequences encoding epitope regions of apolipoprotein B-100 (Smith et al., 1998).

Transgenic mice expressing human apolipoprotein B and fed a high-fat diet were found to develop high plasma cholesterol levels and displayed an 11-fold increase in atherosclerotic lesions over non-transgenic littermates (Kim and Young, *J. Lipid Res.*, 1998, 39, 703-723; Nishina et al., *J. Lipid Res.*, 1990, 31, 859-869).

In addition, transgenic mice expressing truncated forms of human apolipoprotein B have been employed to identify the carboxyl-terminal structural features of ApoB-100 that are required for interactions with apolipoprotein(a) to generate the Lp(a) lipoprotein particle and to investigate structural features of the LDL receptor-binding region of ApoB-100 (Kim and Young, *J. Lipid Res.*, 1998, 39, 703-723; McCormick et al., *J. Biol. Chem.*, 1997, 272, 23616-23622).

Apolipoprotein B knockout mice (bearing disruptions of both ApoB-100 and ApoB-48) have been generated which are protected from developing hypercholesterolemia when fed a high-fat diet (Farese et al., *Proc. Natl. Acad. Sci. U. S. A.*, 1995, 92, 1774-1778; Kim and Young, *J. Lipid Res.*, 1998, 39, 703-723). The incidence of atherosclerosis has been investigated in mice expressing exclusively ApoB-100 or ApoB-48 and susceptibility to atherosclerosis was found to be dependent on total cholesterol levels. Whether the mice synthesized ApoB-100 or ApoB-48 did not affect the extent of the atherosclerosis, indicating that there is probably no major difference in the intrinsic

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atherogenicity of ApoB-100 versus ApoB-48 (Kim and Young, *J. Lipid Res.*, 1998, 39, 703-723; Veniant et al., *J. Clin. Invest.*, 1997, 100, 180-188).

Elevated plasma levels of the ApoB-100-containing  
5 lipoprotein Lp(a) are associated with increased risk for  
atherosclerosis and its manifestations, which may include  
hypercholesterolemia (Seed et al., *N. Engl. J. Med.*, 1990,  
322, 1494-1499), myocardial infarction (Sandkamp et al.,  
*Clin. Chem.*, 1990, 36, 20-23), and thrombosis (Nowak-Gottl  
10 et al., *Pediatrics*, 1997, 99, E11).

The plasma concentration of Lp(a) is strongly  
influenced by heritable factors and is refractory to most  
drug and dietary manipulation (Katan and Beynen, *Am. J.*  
*Epidemiol.*, 1987, 125, 387-399; Vessby et al.,  
15 *Atherosclerosis*, 1982, 44, 61-71). Pharmacologic therapy of  
elevated Lp(a) levels has been only modestly successful and  
apheresis remains the most effective therapeutic modality  
(Hajjar and Nachman, *Annu. Rev. Med.*, 1996, 47, 423-442).

Disclosed and claimed in US patent 6,156,315 and the  
20 corresponding PCT publication WO 99/18986 is a method for  
inhibiting the binding of LDL to blood vessel matrix in a  
subject, comprising administering to the subject an  
effective amount of an antibody or a fragment thereof,  
which is capable of binding to the amino-terminal region of  
25 apolipoprotein B, thereby inhibiting the binding of low  
density lipoprotein to blood vessel matrix (Goldberg and  
Pillarisetti, 2000; Goldberg and Pillarisetti, 1999).

Disclosed and claimed in US patent 6,096,516 are  
vectors containing cDNA encoding murine recombinant  
30 antibodies which bind to human ApoB-100 for the purpose of  
for diagnosis and treatment of cardiovascular diseases  
(Kwak et al., 2000).

Disclosed and claimed in European patent application EP 911344 published April 28, 1999 (and corresponding to U.S. Patent 6,309,844) is a monoclonal antibody which specifically binds to ApoB-48 and does not specifically bind to ApoB-100, which is useful for diagnosis and therapy of hyperlipidemia and arterial sclerosis (Uchida and Kurano, 1998).

Disclosed and claimed in PCT publication WO 01/30354 are methods of treating a patient with a cardiovascular disorder, comprising administering a therapeutically effective amount of a compound to said patient, wherein said compound acts for a period of time to lower plasma concentrations of apolipoprotein B or apolipoprotein B-containing lipoproteins by stimulating a pathway for apolipoprotein B degradation (Fisher and Williams, 2001).

Disclosed and claimed in US patent 5,220,006 is a cloned *cis*-acting DNA sequence that mediates the suppression of atherogenic apolipoprotein B (Ross et al., 1993).

Disclosed and claimed in PCT publication WO 01/12789 is a ribozyme which cleaves ApoB-100 mRNA specifically at position 6679 (Chan et al., 2001).

To date, strategies aimed at inhibiting apolipoprotein B function have been limited to Lp(a) apheresis, antibodies, antibody fragments and ribozymes. However, with the exception of Lp(a) apheresis, these investigative strategies are untested as therapeutic protocols. Consequently, there remains a long felt need for additional agents capable of effectively inhibiting apolipoprotein B function.

Antisense technology is emerging as an effective means of reducing the expression of specific gene products and

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may therefore prove to be uniquely useful in a number of therapeutic, diagnostic and research applications involving modulation of apolipoprotein B expression.

The present invention provides compositions and  
5 methods for modulating apolipoprotein B expression,  
including inhibition of the alternative isoform of  
apolipoprotein B, ApoB-48.

#### SUMMARY OF THE INVENTION

10 The present invention is directed to compounds,  
particularly antisense oligonucleotides, which are targeted  
to a nucleic acid encoding apolipoprotein B, and which  
modulate the expression of apolipoprotein B.

Pharmaceutical and other compositions comprising the  
15 compounds of the invention are also provided. Further  
provided are methods of modulating the expression of  
apolipoprotein B in cells or tissues comprising contacting  
said cells or tissues with one or more of the antisense  
compounds or compositions of the invention. Further  
20 provided are methods of treating an animal, particularly a  
human, suspected of having or being prone to a disease or  
condition associated with expression of apolipoprotein B by  
administering a therapeutically or prophylactically  
effective amount of one or more of the antisense compounds  
25 or compositions of the invention.

In particular, the invention provides a compound 8 to  
50 nucleobases in length targeted to a nucleic acid  
molecule encoding apolipoprotein B, wherein said compound  
specifically hybridizes with and inhibits the expression of  
30 a nucleic acid molecule encoding apolipoprotein B, said  
compound comprising at least 8 contiguous nucleobases of  
any one of SEQ ID NOs: 127-134, 136, 138-174, 176-317, 319-

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321, 323-333, 335-339, 341-374, 376-416, 418-500, 502-510, 512-804, 815, 816, 819-821, 824, 825, 827, 828, 830, 831, 833-835, 837-839, 842, 843, and 845-854.

The invention further provides compound 8 to 50  
5 nucleobases in length which specifically hybridizes with at least an 8-nucleobase portion of an active site on a nucleic acid molecule encoding apolipoprotein B, said compound comprising at least 8 contiguous nucleobases of any one of SEQ ID NOS: 127-134, 136, 138-174, 176-317, 319-  
10 321, 323-333, 335-339, 341-374, 376-416, 418-500, 502-510, 512-804, 815, 816, 819-821, 824, 825, 827, 828, 830, 831, 833-835, 837-839, 842, 843, and 845-854, said active site being a region in said nucleic acid wherein binding of said compound to said site significantly inhibits apolipoprotein  
15 B expression as compared to a control.

The invention also provides a compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding apolipoprotein B, wherein said compound specifically hybridizes with said nucleic acid and inhibits  
20 expression of apolipoprotein B, wherein the apolipoprotein B is encoded by a polynucleotide selected from the group consisting of: (a) SEQ ID NO: 3 and (b) a naturally occurring variant apolipoprotein B-encoding polynucleotide that hybridizes to the complement of the polynucleotide of  
25 (a) under stringent conditions, said compound comprising at least 8 contiguous nucleobases of any one of SEQ ID NOS: 127-134, 136, 138-174, 176-317, 319-321, 323-333, 335-339, 341-374, 376-416, 418-500, 502-510, 512-804, 815, 816, 819-821, 824, 825, 827, 828, 830, 831, 833-835, 837-839, 842,  
30 843, and 845-854.

In another aspect the invention provides a compound 8 to 50 nucleobases in length targeted to a nucleic acid

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molecule encoding apolipoprotein B, wherein said compound specifically hybridizes with said nucleic acid and inhibits expression of apolipoprotein B, wherein the apolipoprotein B is encoded by a polynucleotide selected from the group

5 consisting of SEQ ID NO: 3 and SEQ ID NO: 17, said compound comprising at least 8 contiguous nucleobases of any one of SEQ ID NOS: 127-134, 136, 138-174, 176-317, 319-321, 323-333, 335-339, 341-374, 376-416, 418-500, 502-510, 512-804, 815, 816, 819-821, 824, 825, 827, 828, 830, 831, 833-835,

10 837-839, 842, 843, and 845-854.

The invention also provides a compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding apolipoprotein B, wherein said compound specifically hybridizes with an active site in said nucleic

15 acid and inhibits expression of apolipoprotein B, said compound comprising at least 8 contiguous nucleobases of any one of SEQ ID NOS: 127-134, 136, 138-174, 176-317, 319-321, 323-333, 335-339, 341-374, 376-416, 418-500, 502-510, 512-804, 815, 816, 819-821, 824, 825, 827, 828, 830, 831,

20 833-835, 837-839, 842, 843, and 845-854, said active site being a region in said nucleic acid wherein binding of said compound to said site significantly inhibits apolipoprotein B expression as compared to a control.

In another aspect the invention provides an

25 oligonucleotide mimetic compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding apolipoprotein B, wherein said compound specifically hybridizes with said nucleic acid and inhibits expression of apolipoprotein B, said compound comprising at least 8

30 contiguous nucleobases of any one of SEQ ID NOS: 127-134, 136, 138-174, 176-317, 319-321, 323-333, 335-339, 341-374, 376-416, 418-500, 502-510, 512-804, 815, 816, 819-821, 824,

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825, 827, 828, 830, 831, 833-835, 837-839, 842, 843, and  
845-854.

In one aspect, the compounds of the invention are  
targeted to a nucleic acid molecule encoding apolipoprotein  
5 B, wherein said compound specifically hybridizes with and  
inhibits expression of the long form of apolipoprotein B,  
ApoB-100. In another aspect, the compounds specifically  
hybridizes with said nucleic acid and inhibits expression  
of mRNA encoding apolipoprotein B by at least 5% in 80%  
10 confluent HepG2 cells in culture at an optimum  
concentration. In yet another aspect, the compounds  
inhibits expression of mRNA encoding apolipoprotein B by at  
least 10%, at least 15%, at least 20%, at least 25%, at  
least 30%, at least 35%, at least 40%, or at least 50%.

15 In one aspect, the compounds are antisense  
oligonucleotides, and in one embodiment the compound has a  
sequence comprising SEQ ID NO: 224, the antisense  
oligonucleotide hybridizes with a region complementary to  
SEQ ID NO: 224, the compound comprises SEQ ID NO: 224, the  
20 compound consists essentially of SEQ ID NO: 224 or the  
compound consists of SEQ ID NO: 224.

In another aspect, the compound has a sequence  
comprising SEQ ID NO: 247, the antisense oligonucleotide  
hybridizes with a region complementary to SEQ ID NO: 247,  
25 the compound comprises SEQ ID NO: 247, the compound  
consists essentially of SEQ ID NO: 247 or the compound  
consists of SEQ ID NO: 247.

In another aspect, the compound has a sequence  
comprising SEQ ID NO: 319, the antisense oligonucleotide  
30 hybridizes with a region complementary to SEQ ID NO: 319,  
the compound comprises SEQ ID NO: 319, the compound



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consists essentially of SEQ ID NO: 319 or the compound  
consists of SEQ ID NO: 319.

In one embodiment, the compounds comprise at least one  
modified internucleoside linkage, and in another  
5 embodiment, the modified internucleoside linkage is a  
phosphorothioate linkage.

In another aspect, the compounds comprise at least one  
modified sugar moiety, and in one aspect, the modified  
sugar moiety is a 2'-O-methoxyethyl sugar moiety.

10 In another embodiment, the compounds comprise at least  
one modified nucleobase, and in one aspect, the modified  
nucleobase is a 5-methylcytosine.

In yet another aspect, the compounds are chimeric  
oligonucleotides.

15 In another aspect, the compounds specifically  
hybridizes with and inhibits the expression of a nucleic  
acid molecule encoding an alternatively spliced form of  
apolipoprotein B.

The invention also provide compositions comprising a  
20 compound of the invention and a pharmaceutically acceptable  
carrier or diluent. In one aspect, the composition further  
comprises a colloidal dispersion system, and in another  
aspect, the compound in the composition is an antisense  
oligonucleotide.

25 The invention further provides methods of inhibiting  
the expression of apolipoprotein B in cells or tissues  
comprising contacting said cells or tissues with a compound  
of the invention so that expression of apolipoprotein B is  
inhibited. Methods are also provided for treating an animal  
30 having a disease or condition associated with  
apolipoprotein B comprising administering to said animal a  
therapeutically or prophylactically effective amount of a

compound of the invention so that expression of apolipoprotein B is inhibited. In various aspects, the condition is associated with abnormal lipid metabolism, the condition is associated with abnormal cholesterol metabolism, the condition is atherosclerosis, the condition is an abnormal metabolic condition, the abnormal metabolic condition is hyperlipidemia, the disease is diabetes, the diabetes is Type 2 diabetes, the condition is obesity, and/or the disease is cardiovascular disease.

10       The invention also provide methods of modulating glucose levels in an animal comprising administering to said animal a compound of the invention, and in one aspect, the animal is a human. In various embodiments, the glucose levels are plasma glucose levels, the glucose levels are serum glucose levels, and/or the animal is a diabetic animal.

15       The invention also provides methods of preventing or delaying the onset of a disease or condition associated with apolipoprotein B in an animal comprising administering to said animal a therapeutically or prophylactically effective amount of a compound of the invention. In one aspect, the animal is a human. In other aspects, the condition is an abnormal metabolic condition, the abnormal metabolic condition is hyperlipidemia, the disease is diabetes, the diabetes is Type 2 diabetes, the condition is obesity, the condition is atherosclerosis, the condition involves abnormal lipid metabolism, and/or the condition involves abnormal cholesterol metabolism.

20       The invention also provides methods of preventing or delaying the onset of an increase in glucose levels in an animal comprising administering to said animal a therapeutically or prophylactically effective amount of a

compound of the invention. In one aspect, the animal is a human. In other aspects, the glucose levels are serum glucose levels, and/or the glucose levels are plasma glucose levels.

5           The invention also provides methods of modulating serum cholesterol levels in an animal comprising administering to said animal a therapeutically or prophylactically effective amount of a compound of the invention. In one aspect, the animal is a human.

10           The invention also provides methods of modulating lipoprotein levels in an animal comprising administering to said animal a therapeutically or prophylactically effective amount of a compound of the invention. In one aspect, the animal is a human. In other aspects, the lipoprotein is  
15 VLDL, the lipoprotein is HDL, and/or the lipoprotein is LDL.

          The invention also provides methods of modulating serum triglyceride levels in an animal comprising administering to said animal a therapeutically or  
20 prophylactically effective amount of a compound of the invention. In one aspect, the animal is a human.

          The invention also proves use of a compound of the invention for the manufacture of a medicament for the treatment of a disease or condition associated with  
25 apolipoprotein B expression, a medicament for the treatment of a condition associated with abnormal lipid metabolism, a medicament for the treatment of a condition associated with abnormal cholesterol metabolism, a medicament for the treatment of atherosclerosis, a medicament for the  
30 treatment of hyperlipidemia, a medicament for the treatment of diabetes, a medicament for the treatment of Type 2 diabetes, a medicament for the treatment of obesity, a

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medicament for the treatment of cardiovascular disease, a  
medicament for preventing or delaying the onset of  
increased glucose levels, a medicament for preventing or  
delaying the onset of increased serum glucose levels, a  
5 medicament for preventing or delaying the onset of  
increased plasma glucose levels, a medicament for the  
modulation of serum cholesterol levels, a medicament for  
the modulation of serum lipoprotein levels, a medicament  
for the modulation of serum VLDL levels, a medicament for  
10 the modulation of serum HDL levels, and/or a medicament for  
the modulation of serum LDL levels, a medicament for the  
modulation of serum triglyceride levels.

In another aspect, the invention provides methods of  
decreasing circulating lipoprotein levels comprising the  
15 step of administering to an individual an amount of a  
compound of the invention sufficient to reduce  
apolipoprotein B expression. In another aspect, the  
invention provides methods of reducing lipoprotein  
transport comprising the step of administering to an  
20 individual an amount of a compound of the invention  
sufficient to reduce apolipoprotein B expression. The  
invention also provides methods of reducing lipoprotein  
absorption/adsorption comprising the step of administering  
to an individual an amount of a compound of the invention  
25 sufficient to reduce apolipoprotein B expression.

In another aspect, the invention contemplates methods  
of decreasing circulating triglyceride levels comprising  
the step of administering to an individual an amount of a  
compound of the invention sufficient to reduce  
30 apolipoprotein B expression. Also provided are methods of  
reducing triglyceride transport comprising the step of  
administering to an individual an amount of a compound of

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the invention sufficient to reduce apolipoprotein B expression. The invention further provides methods of reducing triglyceride absorption/adsorption comprising the step of administering to an individual an amount of a  
5 compound of the invention sufficient to reduce apolipoprotein B expression.

In another aspect, the invention provides methods of decreasing circulating cholesterol levels, including cholesteryl esters and/or unesterified cholesterol,  
10 comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. Also contemplated are methods of reducing cholesterol transport, including cholesteryl  
esters and/or unesterified cholesterol, comprising the step  
15 of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. The invention also provides methods of reducing cholesterol absorption/adsorption, including cholesteryl  
esters and/or unesterified cholesterol, comprising the step  
20 of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

In another aspect, the invention provides methods of decreasing circulating lipid levels comprising the step of  
25 administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. The invention also provides methods of reducing lipid transport in plasma comprising the step of  
administering to an individual an amount of a compound of  
30 the invention sufficient to reduce apolipoprotein B expression. In addition, the invention provides methods of reducing lipid absorption/adsorption comprising the step of

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administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

The invention further contemplates methods of decreasing circulating dietary lipid levels comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. Also provided are methods of reducing dietary lipid transport comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression, as well as methods of reducing dietary lipid absorption/adsorption comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

In another aspect, the invention provides methods of decreasing circulating fatty acid levels comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. The invention also provides methods of reducing fatty acid transport comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. Also contemplated are methods of reducing fatty acid absorption comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

The invention also provides methods of decreasing circulating acute phase reactants comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B

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expression. In another aspect, the invention provides methods of reducing acute phase reactants transport comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression, as well as methods of reducing acute phase reactants absorption comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

10 In another aspect, the invention provides methods of decreasing circulating chylomicrons comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression, methods of reducing chylomicron transport  
15 comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression, and methods of reducing chylomicron absorption comprising the step of administering to an individual an amount of a compound of the invention  
20 sufficient to reduce apolipoprotein B expression.

The invention further provides methods of decreasing circulating chylomicron remnant particles comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce  
25 apolipoprotein B expression, methods of reducing chylomicron remnant transport comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression, and methods of reducing chylomicron remnant  
30 absorption comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

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The invention further contemplates methods of decreasing circulating VLDL, IDL, LDL, and/or HDL comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. Likewise, the invention provides methods of reducing VLDL, IDL, LDL, and/or HDL transport comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression, in addition to methods of reducing VLDL, IDL, LDL, and/or HDL absorption comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

In still another aspect, the invention provides methods of treating a condition associated with apolipoprotein B expression comprising the step of administering to an individual an amount of a compound of the invention sufficient to inhibit apolipoprotein B expression, said condition selected from hyperlipoproteinemia, familial type 3 hyperlipoproteinemia (familial dysbetalipoproteinemia), and familial hyperalphalipoproteinemia; hyperlipidemia, mixed hyperlipidemias, multiple lipoprotein-type hyperlipidemia, and familial combined hyperlipidemia; hypertriglyceridemia, familial hypertriglyceridemia, and familial lipoprotein lipase; hypercholesterolemia, familial hypercholesterolemia, polygenic hypercholesterolemia, and familial defective apolipoprotein B; cardiovascular disorders including atherosclerosis and coronary artery disease; peripheral vascular disease; von Gierke's disease (glycogen storage disease, type I); lipodystrophies (congenital and acquired forms); Cushing's syndrome; sexual



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ateloitic dwarfism (isolated growth hormone deficiency); diabetes mellitus; hyperthyroidism; hypertension; anorexia nervosa; Werner's syndrome; acute intermittent porphyria; primary biliary cirrhosis; extrahepatic biliary  
5 obstruction; acute hepatitis; hepatoma; systemic lupus erythematosus; monoclonal gammopathies (including myeloma, multiple myeloma, macroglobulinemia, and lymphoma); endocrinopathies; obesity; nephrotic syndrome; metabolic syndrome; inflammation; hypothyroidism; uremia  
10 (hyperurecemia); impotence; obstructive liver disease; idiopathic hypercalcemia; dysglobulinemia; elevated insulin levels; Syndrome X; Dupuytren's contracture; and Alzheimer's disease and dementia.

The invention also provides methods of reducing the  
15 risk of a condition comprising the step of administering to an individual an amount of a compound of the invention sufficient to inhibit apolipoprotein B expression, said condition selected from pregnancy; intermittent claudication; gout; and mercury toxicity and amalgam  
20 illness.

The invention further provides methods of inhibiting cholesterol particle binding to vascular endothelium comprising the step of administering to an individual an amount of a compound of the invention sufficient to inhibit  
25 apolipoprotein B expression, and as a result, the invention also provides methods of reducing the risk of: (i) cholesterol particle oxidization; (ii) monocyte binding to vascular endothelium; (iii) monocyte differentiation into macrophage; (iv) macrophage ingestion of oxidized lipid  
30 particles and release of cytokines (including, but limited to IL-1, TNF-alpha, TGF-beta); (v) platelet formation of fibrous fibrofatty lesions and inflammation; (vi)

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endothelium lesions leading to clots; and (vii) clots leading to myocardial infarction or stroke, also comprising the step of administering to an individual an amount of a compound of the invention sufficient to inhibit apolipoprotein B expression.

The invention also provides methods of reducing hyperlipidemia associated with alcoholism, smoking, use of oral contraceptives, use of glucocorticoids, use of beta-adrenergic blocking agents, or use of isotretinoin (13-cis-retinoic acid) comprising the step of administering to an individual an amount of a compound of the invention sufficient to inhibit apolipoprotein B expression.

The invention further provides use of a compound of the invention in the manufacture of a medicament for the treatment of any and all conditions disclosed herein.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric compounds, particularly antisense oligonucleotides, for use in modulating the function of nucleic acid molecules encoding apolipoprotein B, ultimately modulating the amount of apolipoprotein B produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding apolipoprotein B.

As used herein, the terms "target nucleic acid" and "nucleic acid encoding apolipoprotein B" encompass DNA encoding apolipoprotein B, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which

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specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of apolipoprotein B. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding apolipoprotein B. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred

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intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in  
5 transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or  
10 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or  
15 formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set  
20 of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding apolipoprotein B, regardless of the sequence(s) of  
25 such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences; i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and  
30 "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to

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about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated)

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regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds.

"Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen

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bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed.

Antisense and other compounds of the invention which hybridize to the target and inhibit expression of the target are identified through experimentation, and the sequences of these compounds are hereinbelow identified as preferred embodiments of the invention. The target sites to which these preferred sequences are complementary are hereinbelow referred to as "active sites" and are therefore preferred sites for targeting. Therefore another embodiment of the invention encompasses compounds which hybridize to these active sites.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression

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with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

For use in kits and diagnostics, the antisense compounds of the present invention, either alone or in combination with other antisense compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and tissues.

Expression patterns within cells or tissues treated with one or more antisense compounds are compared to control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for differential levels of gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds which affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, 2000, 480, 17-24; Celis, et al., *FEBS Lett.*, 2000, 480, 2-16), SAGE (serial analysis of gene expression) (Madden, et al., *Drug Discov. Today*, 2000, 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, 1999, 303, 258-72), TOGA (total gene expression analysis)



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(Sutcliffe, et al., *Proc. Natl. Acad. Sci. U. S. A.*, 2000, 97, 1976-81), protein arrays and proteomics (Celis, et al., *FEBS Lett.*, 2000, 480, 2-16; Jungblut, et al., *Electrophoresis*, 1999, 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, et al., *FEBS Lett.*, 2000, 480, 2-16; Larsson, et al., *J. Biotechnol.*, 2000, 80, 143-57), subtractive RNA fingerprinting (SuRF) (Fuchs, et al., *Anal. Biochem.*, 2000, 286, 91-98; Larson, et al., *Cytometry*, 2000, 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*, 2000, 3, 316-21), comparative genomic hybridization (Carulli, et al., *J. Cell Biochem. Suppl.*, 1998, 31, 286-96), FISH (fluorescent in situ hybridization) techniques (Going and Gusterson, *Eur. J. Cancer*, 1999, 35, 1895-904) and mass spectrometry methods (reviewed in (To, *Comb. Chem. High Throughput Screen*, 2000, 3, 235-41)).

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and

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covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 50 nucleobases (i.e. from about 8 to about 50 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12 to about 30 nucleobases. Antisense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its expression.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups

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covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

10 Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

20 Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thiono-alkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide

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linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide

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backbones; and others having mixed N, O, S and CH<sub>2</sub> component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262; each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, 1991, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular  $-\text{CH}_2-\text{NH}-\text{O}-\text{CH}_2-$ ,  $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{O}-\text{CH}_2-$  [known as a  
5 methylene (methylimino) or MMI backbone],  $-\text{CH}_2-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-$ ,  
 ,  $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{N}(\text{CH}_3)-\text{CH}_2-$  and  $-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-$  [wherein the native phosphodiester backbone is represented as  $-\text{O}-\text{P}-\text{O}-\text{C H}_2-$   
] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent  
10 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides  
15 comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted  $\text{C}_1$  to  $\text{C}_{10}$  alkyl or  $\text{C}_2$  to  $\text{C}_{10}$  alkenyl and alkynyl. Particularly preferred  
20 are  $\text{O}[(\text{CH}_2)_n\text{O}]_m\text{CH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{OCH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{NH}_2$ ,  $\text{O}(\text{CH}_2)_n\text{CH}_3$ ,  
 $\text{O}(\text{CH}_2)_n\text{ONH}_2$ , and  $\text{O}(\text{CH}_2)_n\text{ON}[(\text{CH}_2)_n\text{CH}_3]_2$ , where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position:  $\text{C}_1$  to  $\text{C}_{10}$  lower alkyl, substituted lower alkyl, alkenyl, alkynyl,  
25 alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH,  $\text{SCH}_3$ , OCN, Cl, Br, CN,  $\text{CF}_3$ ,  $\text{OCF}_3$ ,  $\text{SOCH}_3$ ,  $\text{SO}_2\text{CH}_3$ ,  $\text{ONO}_2$ ,  $\text{NO}_2$ ,  $\text{N}_3$ ,  $\text{NH}_2$ , heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the  
30 pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar

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properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoethoxy, i.e., a O(CH<sub>2</sub>)<sub>2</sub>ON(CH<sub>3</sub>)<sub>2</sub> group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>2</sub>)<sub>2</sub>, also described in examples hereinbelow.

A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene (-CH<sub>2</sub>-)<sub>n</sub> group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

Other preferred modifications include 2'-methoxy (2'-O-CH<sub>3</sub>), 2'-aminopropoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>), 2'-allyl (2'-CH<sub>2</sub>-CH=CH<sub>2</sub>), 2'-O-allyl (2'-O-CH<sub>2</sub>-CH=CH<sub>2</sub>) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.:

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4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878;  
5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811;  
5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053;  
5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and  
5 5,700,920, certain of which are commonly owned with the  
instant application, and each of which is herein  
incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often  
referred to in the art simply as "base") modifications or  
10 substitutions. As used herein, "unmodified" or "natural"  
nucleobases include the purine bases adenine (A) and  
guanine (G), and the pyrimidine bases thymine (T), cytosine  
(C) and uracil (U). Modified nucleobases include other  
synthetic and natural nucleobases such as 5-methylcytosine  
15 (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine,  
2-aminoadenine, 6-methyl and other alkyl derivatives of  
adenine and guanine, 2-propyl and other alkyl derivatives  
of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-  
thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-  
20 CH<sub>3</sub>) uracil and cytosine and other alkynyl derivatives of  
pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-  
uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-  
thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted  
adenines and guanines, 5-halo particularly 5-bromo, 5-  
25 trifluoromethyl and other 5-substituted uracils and cyto-  
sines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-  
amino-adenine, 8-azaguanine and 8-azaadenine, 7-  
deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-  
deazaadenine. Further modified nucleobases include  
30 tricyclic pyrimidines such as phenoxazine cytidine (1H-  
pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine  
cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one),



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G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyl-adenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2° C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include,

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but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds of the invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer

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uptake, distribution, metabolism or excretion.

Representative conjugate groups are disclosed in

International Patent Application PCT/US92/09196, filed

October 23, 1992 the entire disclosure of which is incor-

5 porated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4, 1053-1060), a thioether, e.g., hexyl-

10 S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-

15 Behmoaras et al., *EMBO J.*, 1991, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, 1990, 259, 327-330; Svinarchuk et al., *Biochimie*, 1993, 75, 49-54), a phospholipid, e.g., dihexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al.,

20 *Tetrahedron Lett.*, 1995, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654),

25 a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277, 923-937.

Oligonucleotides of the invention may also be conjugated to

30 active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (S)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-

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triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethacin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic.

- 5 Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its entirety.

Representative United States patents that teach the  
10 preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718;  
15 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241;  
20 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein  
25 incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within  
30 an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds.

"Chimeric" antisense compounds or "chimeras," in the

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context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.:

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5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878;  
5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355;  
5,652,356; and 5,700,922, certain of which are commonly  
owned with the instant application, and each of which is  
5 herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this  
invention may be conveniently and routinely made through  
the well-known technique of solid phase synthesis.  
Equipment for such synthesis is sold by several vendors  
10 including, for example, Applied Biosystems (Foster City,  
CA). Any other means for such synthesis known in the art  
may additionally or alternatively be employed. It is well  
known to use similar techniques to prepare oligonucleotides  
such as the phosphorothioates and alkylated derivatives.

15 The antisense compounds of the invention are  
synthesized *in vitro* and do not include antisense  
compositions of biological origin, or genetic vector  
constructs designed to direct the *in vivo* synthesis of  
antisense molecules.

20 The compounds of the invention may also be admixed,  
encapsulated, conjugated or otherwise associated with other  
molecules, molecule structures or mixtures of compounds, as  
for example, liposomes, receptor targeted molecules, oral,  
rectal, topical or other formulations, for assisting in  
25 uptake, distribution and/or absorption. Representative  
United States patents that teach the preparation of such  
uptake, distribution and/or absorption assisting  
formulations include, but are not limited to, U.S.:

5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291;  
30 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330;  
4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170;  
5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978;

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5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259;  
5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of  
which is herein incorporated by reference.

The antisense compounds of the invention encompass any  
5 pharmaceutically acceptable salts, esters, or salts of such  
esters, or any other compound which, upon administration to  
an animal including a human, is capable of providing  
(directly or indirectly) the biologically active metabolite  
or residue thereof. Accordingly, for example, the  
10 disclosure is also drawn to prodrugs and pharmaceutically  
acceptable salts of the compounds of the invention,  
pharmaceutically acceptable salts of such prodrugs, and  
other bioequivalents.

The term "prodrug" indicates a therapeutic agent that  
15 is prepared in an inactive form that is converted to an  
active form (i.e., drug) within the body or cells thereof  
by the action of endogenous enzymes or other chemicals  
and/or conditions. In particular, prodrug versions of the  
oligonucleotides of the invention are prepared as SATE  
20 [(S-acetyl-2-thioethyl) phosphate] derivatives according to  
the methods disclosed in WO 93/24510 to Gosselin et al.,  
published December 9, 1993 or in WO 94/26764 and U.S.  
5,770,713 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to  
25 physiologically and pharmaceutically acceptable salts of  
the compounds of the invention: i.e., salts that retain the  
desired biological activity of the parent compound and do  
not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are  
30 formed with metals or amines, such as alkali and alkaline  
earth metals or organic amines. Examples of metals used as  
cations are sodium, potassium, magnesium, calcium, and the

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like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, 66, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid,



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glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic

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acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and  
5 iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease  
10 or disorder which can be treated by modulating the expression of apolipoprotein B is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount  
15 of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

20 The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding apolipoprotein B, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense  
25 oligonucleotides of the invention with a nucleic acid encoding apolipoprotein B can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means.  
30 Kits using such detection means for detecting the level of apolipoprotein B in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC,

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distearolyphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C<sub>1-10</sub> alkyl ester (e.g. isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999 which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitables. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts include

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chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucolic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium

5 tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate, Preferred fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin,

10 glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (e.g. sodium). Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in

15 combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be

20 delivered orally in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents include

poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates;

25 cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses and starches. Particularly preferred complexing agents include chitosan, N-trimethylchitosan,

30 poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylamino-methylethylene P(TDAE), polyaminostyrene (e.g. p-amino),

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poly(methylcyanoacrylate), poly(ethylcyanoacrylate),  
poly(butylcyanoacrylate), poly(isobutylcyanoacrylate),  
poly(isohexylcyanoacrylate), DEAE-methacrylate, DEAE-  
hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-  
5 dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-  
lactic acid), poly(DL-lactic-co-glycolic acid (PLGA),  
alginate, and polyethyleneglycol (PEG). Oral formulations  
for oligonucleotides and their preparation are described in  
detail in United States applications 08/886,829 (filed July  
10 1, 1997), 09/108,673 (filed July 1, 1998), 09/256,515  
(filed February 23, 1999), 09/082,624 (filed May 21, 1998)  
and 09/315,298 (filed May 20, 1999) each of which is  
incorporated herein by reference in their entirety.

Compositions and formulations for parenteral,  
15 intrathecal or intraventricular administration may include  
sterile aqueous solutions which may also contain buffers,  
diluent and other suitable additives such as, but not  
limited to, penetration enhancers, carrier compounds and  
other pharmaceutically acceptable carriers or excipients.

20 Pharmaceutical compositions of the present invention  
include, but are not limited to, solutions, emulsions, and  
liposome-containing formulations. These compositions may  
be generated from a variety of components that include, but  
are not limited to, preformed liquids, self-emulsifying  
25 solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present  
invention, which may conveniently be presented in unit  
dosage form, may be prepared according to conventional  
techniques well known in the pharmaceutical industry. Such  
30 techniques include the step of bringing into association  
the active ingredients with the pharmaceutical carrier(s)  
or excipient(s). In general the formulations are prepared

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by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

5       The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be  
10       formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

15       In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature  
20       these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the  
25       present invention.

#### Emulsions

      The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are  
30       typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1  $\mu\text{m}$  in diameter (Idson, in *Pharmaceutical Dosage Forms*, Lieberman,

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Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245;

5 Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 301). Emulsions are often biphasic systems

10 comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the

15 resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional

20 components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in

25 emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages

30 that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise



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a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion.

Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the

preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as

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polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of

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ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is

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of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules

5 (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how  
10 to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York,  
15 N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

20 Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol  
25 monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain  
30 alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered

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film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art.

5 The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355,  
10 Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

15 Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides  
20 (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug  
25 absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity  
(Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385; Ho et al., *J. Pharm. Sci.*, 1996, 85, 138-143). Often  
30 microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be

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particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical

5 applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local  
10 cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as  
15 sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present  
20 invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Each of these classes has been discussed  
25 above.

#### Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the  
30 formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and

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the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

5       Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-  
10   cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each  
15   with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes  
20   obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical*  
25   *Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

30       Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological



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membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the  
5 active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over  
10 other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and  
15 hydrophobic, into the skin.

Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been  
20 administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable  
25 complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang et al., *Biochem. Biophys. Res.*  
30 *Commun.*, 1987, 147, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it.

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Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs.

Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., *Journal of Controlled Release*, 1992, 19, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (e.g. as a solution or as an emulsion) were ineffective (Weiner et al., *Journal of Drug Targeting*, 1992, 2, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis et al., *Antiviral Research*, 1992, 18, 259-265).

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Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations

- 5 comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin.
- 10 Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al. *S.T.P. Pharma. Sci.*, 1994, 4, 6, 466).

- Liposomes also include "sterically stabilized"
- 15 liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized
- 20 liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G<sub>M1</sub>, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to
- 25 be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced
- 30 uptake into cells of the reticuloendothelial system (RES) (Allen et al., *FEBS Letters*, 1987, 223, 42; Wu et al., *Cancer Research*, 1993, 53, 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos et al. (*Ann. N.Y. Acad. Sci.*, 1987, 507, 64) reported the ability of monosialoganglioside  $G_{M1}$ , galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon et al. (*Proc. Natl. Acad. Sci. U.S.A.*, 1988, 85, 6949). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside  $G_{M1}$  or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim et al.).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto et al. (*Bull. Chem. Soc. Jpn.*, 1980, 53, 2778) described liposomes comprising a nonionic detergent, 2C<sub>12</sub>15G, that contains a PEG moiety. Illum et al. (*FEBS Lett.*, 1984, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (e.g., PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klivanov et al. (*FEBS Lett.*, 1990, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume et al. (*Biochimica et Biophysica Acta*, 1990, 1029, 91) extended such observations to other PEG-derivatized phospholipids,

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e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG.

Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0

5 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle et al. (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin et al. (U.S. Patent No. 5,213,804 and European  
10 Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin et al.) and in WO 94/20073 (Zalipsky et al.) Liposomes comprising PEG-modified ceramide lipids are described in WO  
15 96/10391 (Choi et al.). U.S. Patent Nos. 5,540,935 (Miyazaki et al.) and 5,556,948 (Tagawa et al.) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids  
20 are known in the art. WO 96/40062 to Thierry et al. discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa et al. discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an  
25 antisense RNA. U.S. Patent No. 5,665,710 to Rahman et al. describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love et al. discloses liposomes comprising antisense oligonucleotides targeted to the raf gene.

30 Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may

be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, e.g. they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan

esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The

5 polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include

10 carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important

15 members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include

20 quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is

25 classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in

30 *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

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## Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92); and perfluorochemical



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emulsions, such as FC-43. Takahashi et al., *J. Pharm. Pharmacol.*, 1988, 40, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, 5 oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-10 dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C<sub>1-10</sub> alkyl esters thereof (e.g., methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in* 15 *Therapeutic Drug Carrier Systems*, 1991, p.92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; El Hariri et al., *J. Pharm. Pharmacol.*, 1992, 44, 651-654).

20 Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, 1996, pp. 25 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for 30 example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate),

- glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate),
- 5 chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydrofusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92;
- 10 Swinyard, Chapter 39 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; Yamamoto et al., *J. Pharm. Exp. Ther.*, 1992, 263, 25;
- 15 Yamashita et al., *J. Pharm. Sci.*, 1990, 79, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that

20 absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal

25 ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, 1993, 618, 315-339).

Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-

30 methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines) (Lee et al., *Critical Reviews in*

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*Therapeutic Drug Carrier Systems*, 1991, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; Buur et al., *J. Control Rel.*, 1990, 14, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.*, 1987, 39, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi et al, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al., PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

## Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*, *Antisense Res. Dev.*, 1995, 5, 115-121; Takakura *et al.*, *Antisense & Nucl. Acid Drug Dev.*, 1996, 6, 177-183).

## Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency,

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etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc.).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for

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non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

#### Pulsatile Delivery

10 The compounds of the present invention may also be administered by pulsatile delivery. "Pulsatile delivery" refers to a pharmaceutical formulations that delivers a first pulse of drug combined with a penetration enhancer and a second pulse of penetration enhancer to promote  
15 absorption of drug which is not absorbed upon release with the first pulse of penetration enhancer.

One embodiment of the present invention is a delayed release oral formulation for enhanced intestinal drug absorption, comprising:

20 (a) a first population of carrier particles comprising said drug and a penetration enhancer, wherein said drug and said penetration enhancer are released at a first location in the intestine; and

(b) a second population of carrier particles  
25 comprising a penetration enhancer and a delayed release coating or matrix, wherein the penetration enhancer is released at a second location in the intestine downstream from the first location, whereby absorption of the drug is enhanced when the drug reaches the second location.

30 Alternatively, the penetration enhancer in (a) and (b) is different.

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This enhancement is obtained by encapsulating at least two populations of carrier particles. The first population of carrier particles comprises a biologically active substance and a penetration enhancer, and the second (and  
5 optionally additional) population of carrier particles comprises a penetration enhancer and a delayed release coating or matrix.

A "first pass effect" that applies to orally administered drugs is degradation due to the action of  
10 gastric acid and various digestive enzymes. One means of ameliorating first pass clearance effects is to increase the dose of administered drug, thereby compensating for proportion of drug lost to first pass clearance. Although this may be readily achieved with i.v. administration by,  
15 for example, simply providing more of the drug to an animal, other factors influence the bioavailability of drugs administered via non-parenteral means. For example, a drug may be enzymatically or chemically degraded in the alimentary canal or blood stream and/or may be impermeable  
20 or semipermeable to various mucosal membranes.

It is also contemplated that these pharmaceutical compositions are capable of enhancing absorption of biologically active substances when administered via the rectal, vaginal, nasal or pulmonary routes. It is also  
25 contemplated that release of the biologically active substance can be achieved in any part of the gastrointestinal tract.

Liquid pharmaceutical compositions of oligonucleotide can be prepared by combining the oligonucleotide with a  
30 suitable vehicle, for example sterile pyrogen free water, or saline solution. Other therapeutic compounds may optionally be included.

The present invention also contemplates the use of solid particulate compositions. Such compositions preferably comprise particles of oligonucleotide that are of respirable size. Such particles can be prepared by, for example, grinding dry oligonucleotide by conventional means, fore example with a mortar and pestle, and then passing the resulting powder composition through a 400 mesh screen to segregate large particles and agglomerates. A solid particulate composition comprised of an active oligonucleotide can optionally contain a dispersant which serves to facilitate the formation of an aerosol, for example lactose.

In accordance with the present invention, oligonucleotide compositions can be aerosolized.

15. Aerosolization of liquid particles can be produced by any suitable means, such as with a nebulizer. See, for example, U.S. Patent No. 4,501,729. Nebulizers are commercially available devices which transform solutions or suspensions into a therapeutic aerosol mist either by means of acceleration of a compressed gas, typically air or oxygen, through a narrow venturi orifice or by means of ultrasonic agitation. Suitable nebulizers include those sold by Blairex<sup>®</sup> under the name PARI LC PLUS, PARI DURA-NEB 2000, PARI-BABY Size, PARI PRONEB Compressor with LC PLUS, PARI WALKHALER Compressor/Nebulizer System, PARI LC PLUS Reusable Nebulizer, and PARI LC Jet+ <sup>®</sup> Nebulizer.

Exemplary formulations for use in nebulizers consist of an oligonucleotide in a liquid, such as sterile, pyrogen free water, or saline solution, wherein the oligonucleotide comprises up to about 40% w/w of the formulation.

30 Preferably, the oligonucleotide comprises less than 20% w/w. If desired, further additives such as preservatives



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(for example, methyl hydroxybenzoate) antioxidants, and flavoring agents can be added to the composition.

Solid particles comprising an oligonucleotide can also be aerosolized using any solid particulate medicament aerosol generator known in the art. Such aerosol generators produce respirable particles, as described above, and further produce reproducible metered dose per unit volume of aerosol. Suitable solid particulate aerosol generators include insufflators and metered dose inhalers. Metered dose inhalers are used in the art and are useful in the present invention.

Preferably, liquid or solid aerosols are produced at a rate of from about 10 to 150 liters per minute, more preferably from about 30 to 150 liters per minute, and most preferably about 60 liters per minute.

Enhanced bioavailability of biologically active substances is also achieved via the oral administration of the compositions and methods of the present invention. The term "bioavailability" refers to a measurement of what portion of an administered drug reaches the circulatory system when a non-parenteral mode of administration is used to introduce the drug into an animal.

Penetration enhancers include, but are not limited to, members of molecular classes such as surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactant molecules. (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Carriers are inert molecules that may be included in the compositions of the present invention to interfere with processes that lead to reduction in the levels of bioavailable drug.

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## Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin,

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dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone,

5 hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine,

10 cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine,

15 teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed. 1987, pp. 1206-1228, Berkow et al., eds., Rahway, N.J. When used with the compounds of the invention, such

20 chemotherapeutic agents may be used individually (e.g., 5-FU and oligonucleotide), sequentially (e.g., 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (e.g., 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and

25 oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the

30 invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-

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antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on  $EC_{50}$ s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy

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to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

5

#### Combination Therapy

The invention also provides methods of combination therapy, wherein one or more compounds of the invention and one or more other therapeutic/prophylactic compounds are administered treat a condition and/or disease state as described herein. In various aspects, the compound(s) of the invention and the therapeutic/prophylactic compound(s) are co-administered as a mixture or administered individually. In one aspect, the route of administration is the same for the compound(s) of the invention and the therapeutic/prophylactic compound(s), while in other aspects, the compound(s) of the invention and the therapeutic/prophylactic compound(s) are administered by a different routes. In one embodiment, the dosages of the compound(s) of the invention and the therapeutic/prophylactic compound(s) are amounts that are therapeutically or prophylactically effective for each compound when administered individually. Alternatively, the combined administration permits use of lower dosages than would be required to achieve a therapeutic or prophylactic effect if administered individually, and such methods are useful in decreasing one or more side effects of the reduced-dose compound.

In one aspect, a compound of the present invention and one or more other therapeutic/prophylactic compound(s) effective at treating a condition are administered wherein both compounds act through the same or different

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mechanisms. Therapeutic/prophylactic compound(s) include, but are not limited to, bile salt sequestering resins (e.g., cholestyramine, colestipol, and colesevelam hydrochloride), HMGCoA-reductase inhibitors (e.g., lovastatin, cerivastatin, prevastatin, atorvastatin, simvastatin, and fluvastatin), nicotinic acid, fibric acid derivatives (e.g., clofibrate, gemfibrozil, fenofibrate, bezafibrate, and ciprofibrate), probucol, neomycin, dextrothyroxine, plant-stanol esters, cholesterol absorption inhibitors (e.g., ezetimibe), implitapide, inhibitors of bile acid transporters (apical sodium-dependent bile acid transporters), regulators of hepatic CYP7a, estrogen replacement therapeutics (e.g., tamoxifen), and anti-inflammatories (e.g., glucocorticoids).

Accordingly, the invention further provides use of a compound of the invention and one or more other therapeutic/prophylactic compound(s) as described herein in the manufacture of a medicament for the treatment and/or prevention of a disease or condition as described herein.

#### Targeted Delivery

In another aspect, methods are provided to target a compound of the invention to a specific tissue, organ or location in the body. Exemplary targets include liver, lung, kidney, heart, and atherosclerotic plaques within a blood vessel. Methods of targeting compounds are well known in the art.

In one embodiment, the compound is targeted by direct or local administration. For example, when targeting a blood vessel, the compound is administered directly to the relevant portion of the vessel from inside the lumen of the vessel, e.g., single balloon or double balloon catheter, or

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through the adventitia with material aiding slow release of the compound, e.g., a pluronic gel system as described by Simons et al., *Nature* 359: 67-70 (1992). Other slow release techniques for local delivery of the compound to a vessel include coating a stent with the compound. Methods of delivery of antisense compounds to a blood vessel are disclosed in U.S. Patent No. 6,159,946, which is incorporated by reference in its entirety.

When targeting a particular tissue or organ, the compound may be administered in or around that tissue or organ. For example, U.S. Patent No. 6,547,787, incorporated herein by reference in its entirety, discloses methods and devices for targeting therapeutic agents to the heart. In one aspect, administration occurs by direct injection or by injection into a blood vessel associated with the tissue or organ. For example, when targeting the liver, the compound may be administered by injection or infusion through the portal vein.

In another aspect, methods of targeting a compound are provided which include associating the compound with an agent that directs uptake of the compound by one or more cell types. Exemplary agents include lipids and lipid-based structures such as liposomes generally in combination with an organ- or tissue-specific targeting moiety such as, for example, an antibody, a cell surface receptor, a ligand for a cell surface receptor, a polysaccharide, a drug, a hormone, a hapten, a special lipid and a nucleic acid as described in U. S. Patent No. 6,495,532, the disclosure of which is incorporated herein by reference in its entirety. U.S. Pat. No. 5,399,331, the disclosure of which is incorporated herein by reference in its entirety, describes the coupling of proteins to liposomes through use of a

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crosslinking agent having at least one maleimido group and an amine reactive function; U.S. Pat. Nos. 4,885,172, 5,059,421 and 5,171,578, the disclosures of which are incorporated herein by reference in their entirety,

5 describe linking proteins to liposomes through use of the glycoprotein streptavidin and coating targeting liposomes with polysaccharides. Other lipid based targeting agents include, for example, micelle and crystalline products as described in U.S. Patent No. 6,217,886, the disclosure of  
10 which is incorporated herein by reference in its entirety.

In another aspect, targeting agents include porous polymeric microspheres which are derived from copolymeric and homopolymeric polyesters containing hydrolyzable ester linkages which are biodegradable, as described in U.S. No.  
15 Patent 4,818,542, the disclosure of which is incorporated herein by reference in its entirety. Typical polyesters include polyglycolic (PGA) and polylactic (PLA) acids, and copolymers of glycolide and L(-lactide) (PGL), which are particularly suited for the methods and compositions of the  
20 present invention in that they exhibit low human toxicity and are biodegradable. The particular polyester or other polymer, oligomer, or copolymer utilized as the microspheric polymer matrix is not critical and a variety of polymers may be utilized depending on desired porosity,  
25 consistency, shape and size distribution. Other biodegradable or bioerodable polymers or copolymers include, for example, gelatin, agar, starch, arabinogalactan, albumin, collagen, natural and synthetic materials or polymers, such as, poly( $\epsilon$ -caprolactone),  
30 poly( $\epsilon$ -caprolactone-CO-lactic acid), poly( $\epsilon$ -caprolactone-CO-glycolic acid), poly( $\beta$ -hydroxy butyric acid), polyethylene oxide, polyethylene, poly(alkyl-2-



cyanoacrylate), (e.g., methyl, ethyl, butyl), hydrogels such as poly(hydroxyethyl methacrylate), polyamides (e.g., polyacrylamide), poly(amino acids) (i.e., L-leucine, L-aspartic acid,  $\beta$ -methyl-L-aspartate,  $\beta$ -benzyl-L-aspartate, glutamic acid), poly(2-hydroxyethyl DL-aspartamide), poly(ester urea), poly(L-phenylalanine/ethylene glycol/1,6-diisocyanatohexane) and poly(methyl methacrylate). The exemplary natural and synthetic polymers suitable for targeted delivery are either readily available commercially or are obtainable by condensation polymerization reactions from the suitable monomers or, comonomers or oligomers.

In still another embodiment, U.S. Patent No. 6,562,864, the disclosure of which is incorporated herein by reference in its entirety, describes catechins, including epi and other carbo-cationic isomers and derivatives thereof, which as monomers, dimers and higher multimers can form complexes with nucleophilic and cationic bioactive agents for use as delivery agents. Catechin multimers have a strong affinity for polar proteins, such as those residing in the vascular endothelium, and on cell/organelle membranes and are particularly useful for targeted delivery of bioactive agents to select sites in vivo. In treatment of vascular diseases and disorders, such as atherosclerosis and coronary artery disease, delivery agents include substituted catechin multimers, including amidated catechin multimers which are formed from reaction between catechin and nitrogen containing moieties such as ammonia.

Other targeting strategies of the invention include ADEPT (antibody-directed enzyme prodrug therapy), GDEPT (gene-directed EPT) and VDEPT (virus-directed EPT) as

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described in U.S. Patent No. 6,433,012, the disclosure of which is incorporated herein by reference in its entirety.

The present invention further provides medical devices and kits for targeted delivery, wherein the device is, for example, a syringe, stent, or catheter. Kits include a device for administering a compound and a container comprising a compound of the invention. In one aspect, the compound is preloaded into the device. In other embodiments, the kit provides instructions for methods of administering the compound and dosages. U.S. patents describing medical devices and kits for delivering antisense compounds include US Patent Nos. 6,368,356; 6,344,035; 6,344,028; 6,287,285; 6,200,304; 5,824,049; 5,749,915; 5,674,242; 5,670,161; 5,609,629; 5,593,974; and 5,470,307 (all incorporated herein by reference in their entirety).

While the present invention has been described with specificity in accordance with certain embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

**EXAMPLES****Example 1****Nucleoside Phosphoramidites for Oligonucleotide Synthesis****5 Deoxy and 2'-alkoxy amidites**

2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are  
10 prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was  
15 increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, 1993, 21, 3197-3203] using commercially available  
20 phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

**2'-Fluoro amidites****2'-Fluorodeoxyadenosine amidites**

25 2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, 1993, 36, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was  
30 synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro

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atom is introduced by a  $S_N2$ -displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

#### 10 2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups.

Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

#### 2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'-phosphoramidites.

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**2'-Fluorodeoxycytidine**

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

**2'-O-(2-Methoxyethyl) modified amidites**

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, 1995, 78, 486-504.

**2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]**

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a

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gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

### 2'-O-Methoxyethyl-5-methyluridine

5        2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the  
10        solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH<sub>3</sub>CN (600 mL) and evaporated. A silica gel  
15        column (3 kg) was packed in CH<sub>2</sub>Cl<sub>2</sub>/acetone/MeOH (20:5:3) containing 0.5% Et<sub>3</sub>NH. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional  
20        material was obtained by reworking impure fractions.

### 2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue  
25        dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one  
30        hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with

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CH<sub>3</sub>CN (200 mL). The residue was dissolved in CHCl<sub>3</sub> (1.5 L) and extracted with 2x500 mL of saturated NaHCO<sub>3</sub> and 2x500 mL of saturated NaCl. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated. 275 g of residue was obtained.

5 The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1) containing 0.5% Et<sub>3</sub>NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total  
10 yield of 183 g (57%).

**3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine**

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine  
15 (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the  
20 addition of MeOH. Upon completion of the reaction, as judged by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in CHCl<sub>3</sub> (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water  
25 layers were back extracted with 200 mL of CHCl<sub>3</sub>. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product fractions  
30 were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

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**3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine**

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH<sub>3</sub>CN (700 mL) and set  
5 aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH<sub>3</sub>CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. POCl<sub>3</sub> was added dropwise, over a 30 minute period, to the stirred  
10 solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the  
15 reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO<sub>3</sub> and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was  
20 triturated with EtOAc to give the title compound.

**2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine**

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M)  
25 in dioxane (500 mL) and NH<sub>4</sub>OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400  
30 mL) saturated with NH<sub>3</sub> gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue



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was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

5

**N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine**

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl<sub>3</sub> (700 mL) and extracted with saturated NaHCO<sub>3</sub> (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO<sub>4</sub> and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et<sub>3</sub>NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

**N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite**

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO<sub>3</sub> (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-

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extracted with  $\text{CH}_2\text{Cl}_2$  (300 mL), and the extracts were combined, dried over  $\text{MgSO}_4$  and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting solvent. The pure  
5 fractions were combined to give 90.6 g (87%) of the title compound.

2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites

10

2'-(Dimethylaminooxyethoxy) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites] are prepared as described in the following  
15 paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

20

5'-O-tert-Butyldiphenylsilyl- $\text{O}^2$ -2'-anhydro-5-methyluridine

$\text{O}^2$ -2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500  
25 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. tert-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC ( $R_f$  0.22, ethyl acetate) indicated a  
30 complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium

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bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10° C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O<sup>2</sup>-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The

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product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product.

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butylldiphenylsilyl-5-methyluridine

5'-O-tert-Butylldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with

triphenylphosphine (11.63g, 44.36mmol) and N-

hydroxyphthalimide (7.24g, 44.36mmol). It was then dried

over P<sub>2</sub>O<sub>5</sub> under high vacuum for two days at 40° C. The

reaction mixture was flushed with argon and dry THF

(369.8mL, Aldrich, sure seal bottle) was added to get a

clear solution. Diethyl-azodicarboxylate (6.98mL,

44.36mmol) was added dropwise to the reaction mixture. The

rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop.

After the addition was complete, the reaction was stirred

for 4 hrs. By that time TLC showed the completion of the

reaction (ethylacetate:hexane, 60:40). The solvent was

evaporated in vacuum. Residue obtained was placed on a

flash column and eluted with ethyl acetate:hexane (60:40),

to get 2'-O-([2-phthalimidoxy)ethyl]-5'-t-

butylldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

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5'-O-tert-butylldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butylldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was filtered, the filtrate was washed with ice cold CH<sub>2</sub>Cl<sub>2</sub> and the combined organic phase was washed with water, brine and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solution was concentrated to get 2'-O-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was stirred for 1 h. Solvent was removed under vacuum; residue chromatographed to get 5'-O-tert-butylldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine as white foam (1.95 g, 78%).

5'-O-tert-Butylldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine

5'-O-tert-butylldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10° C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Aqueous NaHCO<sub>3</sub> solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in

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MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10° C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10° C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% NaHCO<sub>3</sub> (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH<sub>2</sub>Cl<sub>2</sub> to get 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

#### 2'-O-(dimethylaminooxyethyl)-5-methyluridine

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub> to get 2'-O-(dimethylaminooxyethyl)-5-methyluridine (766mg, 92.5%).

#### 5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine

2'-O-(dimethylaminooxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P<sub>2</sub>O<sub>5</sub> under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine

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(20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub> (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was added and dried over P<sub>2</sub>O<sub>5</sub> under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-

N,N,N<sup>1</sup>,N<sup>1</sup>-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was

dissolved in ethyl acetate (70mL) and washed with 5% aqueous NaHCO<sub>3</sub> (40mL). Ethyl acetate layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

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**2'-(Aminooxyethoxy) nucleoside amidites**

2'-(Aminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(aminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs.

5 Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

**N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-  
5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-  
10 N,N-diisopropylphosphoramidite]**

The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinasso, C. J.,  
20 WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide  
25 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-hydroxyethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-([2-phthalamidoxy]ethyl)-5'-O-(4,4'-  
30 dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].



2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside  
amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also  
5 known in the art as 2'-O-dimethylaminoethoxyethyl, i.e.,  
2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>2</sub>)<sub>2</sub>, or 2'-DMAEOE nucleoside amidites) are  
prepared as follows. Other nucleoside amidites are prepared  
similarly.

10 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50  
mmol) is slowly added to a solution of borane in tetra-  
hydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL  
bomb. Hydrogen gas evolves as the solid dissolves. O<sup>2</sup>-, 2'-  
15 anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium  
bicarbonate (2.5 mg) are added and the bomb is sealed,  
placed in an oil bath and heated to 155° C for 26 hours. The  
bomb is cooled to room temperature and opened. The crude  
solution is concentrated and the residue partitioned  
20 between water (200 mL) and hexanes (200 mL). The excess  
phenol is extracted into the hexane layer. The aqueous  
layer is extracted with ethyl acetate (3x200 mL) and the  
combined organic layers are washed once with water, dried  
over anhydrous sodium sulfate and concentrated. The  
25 residue is columned on silica gel using methanol/methylene  
chloride 1:20 (which has 2% triethylamine) as the eluent.  
As the column fractions are concentrated a colorless solid  
forms which is collected to give the title compound as a  
white solid.

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5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl]]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (2x200 mL). The combined CH<sub>2</sub>Cl<sub>2</sub> layers are washed with saturated NaHCO<sub>3</sub> solution, followed by saturated NaCl solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using MeOH:CH<sub>2</sub>Cl<sub>2</sub>:Et<sub>3</sub>N (20:1, v/v, with 1% triethylamine) gives the title compound.

5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl]]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH<sub>2</sub>Cl<sub>2</sub> (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

## Example 2

### Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA

synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55° C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

### Example 3

#### Oligonucleoside Synthesis

10 Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked  
15 oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023,  
20 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

25 Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

### Example 4

#### 30 PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to

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in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, 1996, 4, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, 5. herein incorporated by reference.

### Example 5

#### Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed 10 oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" 15 segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

20

#### [2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligo- 25 nucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O- 30 methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s

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repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then

5 lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is  
10 then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

15

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)]  
Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were  
20 prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric  
25 Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester)]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester]  
chimeric oligonucleotides are prepared as per the above  
30 procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidization with iodine to generate

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the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,4-dithiolane-2-one 1,1-dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

10

#### Example 6

#### Oligonucleotide Isolation

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by <sup>31</sup>P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* **1991**, 266, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

30

**Example 7****Oligonucleotide Synthesis - 96 Well Plate Format**

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,4-dihydro-2H-benzodithiole-3-one 1,1-dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl-diisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base-protected beta-cyanoethyl-diisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated  $\text{NH}_4\text{OH}$  at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

**Example 8****Oligonucleotide Analysis - 96 Well Plate Format**

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for



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individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

#### 10 Example 9

##### Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following 7 cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for example Northern blot analysis, Ribonuclease protection assays, or RT-PCR.

##### T-24 cells:

25 The human transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life

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Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

10

#### A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

#### NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

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## HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

## 10 HepG2 cells:

The human hepatoblastoma cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA). HepG2 cells were routinely cultured in Eagle's MEM supplemented with 10% fetal calf serum, non-essential amino acids, and 1 mM sodium pyruvate (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

25

## AML12 cells:

The AML12 (alpha mouse liver 12) cell line was established from hepatocytes from a mouse (CD1 strain, line MT42) transgenic for human TGF alpha. Cells are cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, and 40 ng/ml dexamethasone,

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and 90%; 10% fetal bovine serum. For subculturing, spent medium is removed and fresh media of 0.25% trypsin, 0.03% EDTA solution is added. Fresh trypsin solution (1 to 2 ml) is added and the culture is left to sit at room temperature until the cells detach.

Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

#### Primary mouse hepatocytes:

Primary mouse hepatocytes were prepared from CD-1 mice purchased from Charles River Labs (Wilmington, MA) and were routinely cultured in Hepatocyte Attachment Media (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco/Life Technologies, Gaithersburg, MD), 250nM dexamethasone (Sigma), and 10nM bovine insulin (Sigma). Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 10000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells are plated onto 100 mm or other standard tissue culture plates coated with rat tail collagen (200ug/mL) (Becton Dickinson) and treated similarly using appropriate volumes of medium and oligonucleotide.

#### Hep3B cells:

The human hepatocellular carcinoma cell line Hep3B was obtained from the American Type Culture Collection

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(Manassas, VA). Hep3B cells were routinely cultured in Dulbeccos's MEM high glucose supplemented with 10% fetal calf serum, L-glutamine and pyridoxine hydrochloride (Gibco/Life Technologies, Gaithersburg, MD). Cells were

5 routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 24-well plates (Falcon-Primaria #3846) at a density of 50,000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be

10 seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

#### Rabbit primary hepatocytes:

15 Primary rabbit hepatocytes were purchased from Invitro Technologies (Gaithersburg, MD) and maintained in Dulbecco's modified Eagle's medium (Gibco). When purchased, the cells had been seeded into 96-well plates for use in RT-PCR analysis and were confluent.

20 For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly using appropriate volumes of medium and oligonucleotide.

#### 25 Treatment with antisense compounds:

When cells reached 80% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200  $\mu$ L OPTI-MEM<sup>TM</sup>-1 reduced-serum medium (Gibco BRL) and then treated with 130  $\mu$  L of

30 OPTI-MEM<sup>TM</sup>-1 containing 3.75  $\mu$ g/mL LIPOFECTIN<sup>TM</sup> (Gibco BRL) and the desired concentration of oligonucleotide. After 4-7 hours of treatment, the medium was replaced with fresh

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medium. Cells were harvested 16-24 hours after oligonucleotide treatment.

The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is ISIS 13920, **TCCGTCATCGCTCCTCAGGG**, SEQ ID NO: 1, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to human H-ras. For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCATTCTGCCCCCAAGGA**, SEQ ID NO: 2, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control oligonucleotide that results in 80% inhibition of c-Ha-ras (for ISIS 13920) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest concentration of positive control oligonucleotide that results in 60% inhibition of H-ras or c-raf mRNA is then utilized as the oligonucleotide screening concentration in subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is deemed as unsuitable for oligonucleotide transfection experiments.

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## Example 10

## Analysis of oligonucleotide inhibition of apolipoprotein B expression

Antisense modulation of apolipoprotein B expression

5 can be assayed in a variety of ways known in the art. For example, apolipoprotein B mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can  
10 be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Northern blot analysis is  
15 routine in the art and is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., 1996. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence  
20 Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions.

Protein levels of apolipoprotein B can be quantitated in a variety of ways well known in the art, such as  
25 immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to apolipoprotein B can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation,  
30 Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel,

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F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., 1997.

Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in*

5 *Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., 1997.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp.

10 10.16.1-10.16.11, John Wiley & Sons, Inc., 1998. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-

15 10.8.21, John Wiley & Sons, Inc., 1997. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-

11.2.22, John Wiley & Sons, Inc., 1991.

## 20 Example 11

### Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura et al., *Clin. Chem.*, 1996, 42, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example,

25 Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was

30 washed with 200  $\mu$ L cold PBS. 60  $\mu$ L lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room



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temperature for five minutes. 55  $\mu$ L of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200  $\mu$ L of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60  $\mu$ L of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70° C was added to each well, the plate was incubated on a 90° C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

## Example 12

### Total RNA Isolation

Total RNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200  $\mu$ L cold PBS. 100  $\mu$ L Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100  $\mu$ L of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15

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seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate  
5 was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 µL water into each well, incubating 1 minute,  
10 and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 µL water.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on  
15 the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

### Example 13

#### 20 Real-time Quantitative PCR Analysis of apolipoprotein B mRNA Levels

Quantitation of apolipoprotein B mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied  
25 Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which  
30 amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are

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quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye

5 (e.g., JOE, FAM, or VIC, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon

10 Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe.

When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye.

During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-

15 exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is

20 generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel

25 reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

Prior to quantitative PCR analysis, primer-probe sets  
30 specific to the target gene being measured are evaluated for their ability to be "multiplexed" with a GAPDH amplification reaction. In multiplexing, both the target

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gene and the internal standard gene GAPDH are amplified concurrently in a single sample. In this analysis, mRNA isolated from untreated cells is serially diluted. Each dilution is amplified in the presence of primer-probe sets specific for GAPDH only, target gene only ("single-plexing"), or both (multiplexing). Following PCR amplification, standard curves of GAPDH and target mRNA signal as a function of dilution are generated from both the single-plexed and multiplexed samples. If both the slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10% of their corresponding values generated from the single-plexed samples, the primer-probe set specific for that target is deemed multiplexable. Other methods of PCR are also known in the art.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25  $\mu$ L PCR cocktail (1x TAQMAN<sup>TM</sup> buffer A, 5.5 mM MgCl<sub>2</sub>, 300  $\mu$ M each of dATP, dCTP and dGTP, 600  $\mu$ M of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNase inhibitor, 1.25 Units AMPLITAQ GOLD<sup>TM</sup>, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25  $\mu$ L total RNA solution. The RT reaction was carried out by incubation for 30 minutes at 48° C. Following a 10 minute incubation at 95° C to activate the AMPLITAQ GOLD<sup>TM</sup>, 40 cycles of a two-step PCR protocol were carried out: 95° C for 15 seconds (denaturation) followed by 60° C for 1.5 minutes (annealing/extension).

Gene target quantities obtained by real time RT-PCR are normalized using either the expression level of GAPDH, a gene whose expression is constant, or by quantifying

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total RNA using RiboGreen™ (Molecular Probes, Inc. Eugene, OR). GAPDH expression is quantified by real time RT-PCR, by being run simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RiboGreen™ RNA  
5 quantification reagent from Molecular Probes. Methods of RNA quantification by RiboGreen™ are taught in Jones, L.J., et al, *Analytical Biochemistry*, 1998, 265, 368-374.

In this assay, 175 µL of RiboGreen™ working reagent (RiboGreen™ reagent diluted 1:2865 in 10mM Tris-HCl, 1 mM  
10 EDTA, pH 7.5) is pipetted into a 96-well plate containing 25uL purified, cellular RNA. The plate is read in a CytoFluor 4000 (PE Applied Biosystems) with excitation at 480nm and emission at 520nm.

Probes and primers to human apolipoprotein B were  
15 designed to hybridize to a human apolipoprotein B sequence, using published sequence information (GenBank accession number NM\_000384, incorporated herein as SEQ ID NO: 3).

For human apolipoprotein B the PCR primers were:

forward primer: TGCTAAAGGCACATATGGCCT (SEQ ID NO: 4)  
20 reverse primer: CTCAGGTGGACTCTCCATTGAG (SEQ ID NO: 5) and the PCR probe was: FAM-CTTGTCAGAGGGATCCTAACACTGGCCG-TAMRA (SEQ ID NO: 6) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

25 For human GAPDH the PCR primers were:

forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 7)  
reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO: 8) and the  
PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC-TAMRA 3' (SEQ ID  
NO: 9) where JOE (PE-Applied Biosystems, Foster City, CA)  
30 is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

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Probes and primers to mouse apolipoprotein B were designed to hybridize to a mouse apolipoprotein B sequence, using published sequence information (GenBank accession number M35186, incorporated herein as SEQ ID NO: 10). For

5 mouse apolipoprotein B the PCR primers were:

forward primer: CGTGGGCTCCAGCATTCTA (SEQ ID NO: 11)

reverse primer: AGTCATTTCTGCCTTTGCGTC (SEQ ID NO: 12) and

the PCR probe was: FAM-CCAATGGTCGGGCACTGCTCAA-TAMRA

10 SEQ ID NO: 13) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

For mouse GAPDH the PCR primers were:

forward primer: GGCAAATTCAACGGCACAGT (SEQ ID NO: 14)

reverse primer: GGGTCTCGCTCCTGGAAGAT (SEQ ID NO: 15) and the

15 PCR probe was: 5' JOE-AAGGCCGAGAATGGGAAGCTTGTCATC-TAMRA 3'

(SEQ ID NO: 16) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

#### 20 Example 14

##### Northern blot analysis of apolipoprotein B mRNA levels

Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RNAzol™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA

25 was prepared following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc., Solon, OH). RNA was transferred from the gel to HYBOND™-N+

30 nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B")

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Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc, La Jolla, CA) and then probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's  
5 recommendations for stringent conditions.

To detect human apolipoprotein B, a human apolipoprotein B specific probe was prepared by PCR using the forward primer TGCTAAAGGCACATATGGCCT (SEQ ID NO: 4) and  
10 the reverse primer CTCAGGTTGGACTCTCCATTGAG (SEQ ID NO: 5). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

15 To detect mouse apolipoprotein B, a human apolipoprotein B specific probe was prepared by PCR using the forward primer CGTGGGCTCCAGCATTCTA (SEQ ID NO: 11) and the reverse primer AGTCATTTCTGCCTTTGCGTC (SEQ ID NO: 12). To normalize for variations in loading and transfer  
20 efficiency membranes were stripped and probed for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3  
25 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.